BACTERIAL MEMBRANE FRACTIONS WITH ADJUVANT EFFECT

The present invention relates to the use of a membrane fraction of gram-negative bacteria, in particular of Klebsiella *pneumoniae*, combined with an antigen or hapten, for the preparation of a pharmaceutical composition intended for orienting the immune response toward a Th1 type and/or mixed Th1/Th2 type response directed against said antigen or hapten. This invention comprises, in addition, methods for the preparation of said membrane fractions and the pharmaceutical compositions containing them and their applications to the prevention and treatment of infectious diseases, in particular infections caused by paramyxoviruses such as RSV, and cancers, in particular those whose tumors are associated with tumor antigens.

Vaccination is an effective means for preventing or reducing in particular infections. The success of vaccination campaigns in this field has made it possible to extend the concept of vaccines to the fields of autoimmune diseases, cancer and fertility. On the other hand, vaccinating antigens alone are not always capable of inducing a rapid and sustained antibody response, which requires the presence of adjuvants, that is to say compounds which help (from the latin *adjuvare*: to help) them to induce such responses.

Adjuvants constitute a group of varied compounds with respect to their structure and their origin. There are thus, inter alia, in this category water-in-oil (incomplete Freund's adjuvant) or oil-in-water emulsions, compounds of bacterial origin such as lipopolysaccharide derivatives from Gram-negative bacteria and aluminum salts. Currently, only aluminum salts are used in humans as adjuvant for vaccine preparations.

The development of an antibody response directed against an antigen requires a series of complex events. It involves cells presenting the antigen, regulatory T lymphocytes (Th for T "helper"), and antibody-producing B lymphocytes. Two types of Th lymphocytes may be distinguished according to the profile of cytokines produced: type 1 Th lymphocytes producing IFN-γ and IL-2 and promoting the formation of IgG2a in mice, and type 2 Th lymphocytes producing IL-4, IL-5 and IL-10 with formation of IgG1 in mice (Mosmann, T.R. and Sad S. Immunol. Today 1996, 17:138). Moreover, it has been shown that, for the same given antigen, it is the adjuvant which orients toward the predominant isotype during the antibody response (Toellner K.-M. et al. J. Exp. Med. 1998, 187:1193). Thus, it is known that aluminum salts, such as Alhydrogel, induce, in mice, an essentially Th2 type response and promote the formation of IgG1 or even of IgE (Allison A.C. In Vaccine design - The role of cytokine networks Vol. 293, 1-9 Plenum Press 1997), which can pose problems in subjects with an allergic predisposition. Furthermore, according to the therapeutic target envisaged, a Th1 or mixed (Th1/Th2) type response may be desired.

Thus, there is currently a need to have available novel adjuvants capable of inducing an immune response of the Th1 or mixed (Th1/Th2) type, preferably a mixed Th1/Th2 response for which the Th1 response is close to or greater than the Th2 response.

Surprisingly, the authors of the present invention have demonstrated particular properties of the membrane fraction of a gram-negative bacterium Klebsiella *pneumoniae* (called FMKp), in particular membrane fractions obtained by methods as described below in the examples. The authors have indeed discovered that said membrane fraction FMKp, combined with an antigen, not only had the capacity to increase the antibody response directed against said antigen but also had the capacity

to reorient the cytokine response toward a Th1/Th2 profile, thus corresponding to the particular adjuvant activity sought, this being regardless of the mode of administration of said membrane fractions.

Thus, the subject of the present invention is the use of a membrane fraction of gram-negative bacteria, in particular of Klebsiella *pneumoniae*, combined with an antigen or hapten for orienting the immune response toward a Th1 type and/or mixed Th1/Th2 type response directed against said antigen or hapten, or for the preparation of a pharmaceutical composition intended for orienting the immune response toward a Th1 type and/or mixed Th1/Th2 type response directed against said antigen or hapten.

By orientation of the immune response toward a Th1 and/or mixed Th1/Th2 type response, there is preferred in particular orientation of the immune response which promotes the induction of a Th1 response relative to the Th1/Th2 response obtained with the alum adjuvant.

By orientation of the immune response toward a Th1 and/or mixed Th1/Th2 type response, there is more particularly preferred an orientation of the immune response which increases the titer of IgG2a antibodies directed against the associated antigen by a factor of at least 10, preferably of at least 25, 50 and 100 relative to the IgG2a titer obtained with the alum adjuvant.

In a particularly preferred manner, the immune response is oriented toward a Th1 and/or mixed Th1/Th2 type response in which the Th1 response is close to or greater than the Th2 response. The expression "close to" will be understood to mean a response which, when expressed as titer of IgG2a antibodies directed against the associated antigen, is at least equal to 0.5 times, preferably at least equal to 0.75 times, the

titer of IgG1 antibody directed against said antigen, with a titer of IgG antibody directed against the associated antigen close to or greater than the titer of IgG antibody directed against the associated antigen obtained with the alum or Freund's adjuvant.

The invention also relates to the use according to the invention, characterized in that the membrane fraction comprises at least membrane fractions of two different strains of bacteria.

The expression membrane fraction of a bacterium is understood to mean, in the present invention, any purified or partially purified membrane fraction or extract obtained from a culture of said bacterium and whose method of preparation comprises at least a step of lysing the bacteria obtained after culture and a step of separating the fraction containing the membranes of said bacteria from the total lysate obtained after the lysis step, in particular by centrifugation or filtration.

The expression membrane fraction of the bacterium when said bacterium is Klebsiella *pneumoniae* is also understood to mean, in the present invention, protein P40, an active fraction of the membrane fraction of Klebsiella *pneumoniae*, having an amino acid sequence SEQ ID No. 2, or one of its fragments.

According to the invention, the membrane fractions may be prepared according to methods known to a person skilled in the art, such as for example the method described by Haeuw J.F. et al. (Eur. J. Biochem, 255, 446-454, 1998).

According to a particular embodiment, the invention relates to a use according to the invention, characterized in that the membrane fraction is prepared by a method comprising the following steps:

a) culture of said bacteria in a culture medium allowing their growth followed by centrifugation of said culture;

b) where appropriate, deactivation of the lytic enzymes of the bacterial pellet obtained in step a), followed by centrifugation of the suspension obtained;

c) extraction and removal of nonmembrane proteins and of nucleic acids from the pellet obtained in step a) or b) by at least one cycle of washing the pellet in an extraction solution;

d) digestion of the membrane pellet obtained in step c) in the presence of proteolytic enzymes, followed by centrifugation;

e) at least one cycle of washing of the pellet obtained in step d) in physiological saline and/or in distilled water; and

f) ultrasonication of the pellet obtained in step e).

Step b) of deactivation of the lytic enzymes of the bacterial pellet obtained in step a) may be carried out by any known methods of deactivation of enzymes, such as in particular by heating the resuspended bacterial pellet at a temperature preferably of close to 100°C or by adding an inhibitor of the activity of these enzymes.

Step c) of extraction and removal of the nonmembrane proteins and of the nucleic acids from the pellet obtained in step a) or b) may be carried out, for example, by at least one cycle of washing of the pellet in an extraction solution corresponding to the addition of a hypertonic solution (extraction solution), preferably a saline solution having a molarity of close to 1 M, followed, after a contact period which is sufficient for the desired effect, by centrifugation of the suspension obtained and removal of the supernatant obtained after said centrifugation, it being possible for this washing cycle to be repeated several times.

Step d) of digestion of the membrane pellet obtained in step c) may be carried out in the presence of a solution of proteolytic enzymes such as, for example, trypsin, chymotrypsin, or any known enzyme with proteolytic activity, the reaction conditions, pH of the solution, temperature and duration of the reaction, being preferably adjusted to the optimum conditions for the activity of the chosen enzyme(s), followed by centrifugation, it being possible for this digestion cycle to be repeated several times with the same enzyme, the same combination of enzymes or with a different enzyme for each digestion cycle performed.

Step e) of washing the pellet obtained in step d) is carried out by taking up the pellet in physiological saline or in distilled water followed, after a sufficient period of contact, by centrifugation, it being possible for this washing cycle to be repeated several times.

Finally step f) of ultrasonication of the pellet is intended in particular to disintegrate and homogenize the membrane fraction obtained at the end of step e). The ultrasonication conditions (duration and intensity) will be determined by persons skilled in the art, for example, according to the quantity of membrane fraction to be treated.

According to another particular embodiment, the invention relates to a use according to the invention, characterized in that the membrane fraction is prepared by a method comprising the following steps:

a) culture of said bacteria in a culture medium allowing their growth, followed, where appropriate, by centrifugation;
b) freezing of the culture medium or of the pellet obtained in step a) followed by thawing and drying of the cells;

c) removal, by means of a DNase, of the nucleic acids from the dry cells obtained in step b) which have been resuspended;

d) grinding of the cells obtained in step c) and clarification of the suspension obtained;

e) precipitation, in an acid medium, of the suspension obtained in step d) and removal of the pellet;

f) neutralization of the supernatant obtained in step e) containing the membrane suspension, followed by dialysis and concentration of the membrane suspension; and

g) sterilization of the concentrated membrane suspension obtained in step f).

The thawing conditions in step b) of the method below will of course be determined by persons skilled in the art according to the initial quantity of pellet to be treated, preferably carried out at 4°C for at least 48 hours for the equivalent of 1 kg of dry cells.

In step c), the removal of the nucleic acids is carried out, for example, by the addition of a DNase, at a final concentration of 5 mg/ml of a cell suspension at a concentration equivalent to 5% of dry cells.

The grinding of the cells obtained in step c) may be carried out by means of any system or apparatus known to a person skilled in the art for grinding cells, such as presses or preferably such as grinding in a Manton Gaulinet loop for 30 minutes.

The clarification of the suspension obtained after grinding may be carried out by means of any system or apparatus known to a person skilled in the art for the clarification of ground products of bacterial cells such as the Sharpless system.

Step e) of precipitation in acid medium of the suspension obtained in step d) may be carried out, for example, with acetic acid. The precipitation is followed by the removal of the pellet by means of a Sharpless-type system and by recovering of the supernatant.

Step f) consists in a step in which the supernatant, obtained after precipitation in acid medium, is neutralized, diluted, dialyzed and then concentrated.

Finally, the last step consists in a step of sterilizing the membrane fraction concentrate obtained in the preceding step such as, for example, by heating at 121°C for about 35 minutes.

The invention particularly relates to the use according to the invention, characterized in that the membrane fraction is the Klebsiella *pneumoniae* protein P40 having the sequence SEQ ID No. 2, or one of these fragments.

The expression protein P40 fragment is understood to mean in particular any fragment having an amino acid sequence contained in the amino acid sequence of protein P40 capable of increasing a nonspecific immune response and/or capable of inducing an antitumor immune response, and comprising at least 5 amino acids, preferably at least 10 amino acids and more preferably at least 15 amino acids.

Of course, said protein P40, or its fragments, may be obtained by chemical synthesis or in the form of recombinant peptides.

The invention particularly relates to the use according to the invention, characterized in that said antigen or hapten is chosen from the antigens or haptens specific to an infectious agent, such as a virus, a bacterium, a

fungus or a parasite, or from the antigens associated with tumor cells.

According to the invention, said antigens or haptens are preferably chosen from peptides, lipopeptides, polysaccharides, oligosaccharides, nucleic acids, lipids or any compound capable of specifically directing the immune response toward a Th1 type and/or mixed Th1/Th2 type response against an antigen or hapten specific to an infectious agent or an antigen associated with a tumor cell.

Of course, said antigen or hapten, when it is of a peptide nature, may be obtained by chemical or recombinant synthesis.

The methods for preparing recombinant peptides are nowadays well known to persons skilled in the art and will not be developed in the present description. Among the cells which may be used for the production of these recombinant peptides, there may of course be mentioned bacterial cells (Olins P.O. and Lee S.C., 1993, Recent advances in heterologous gene expression in E. coli Curr. Op. Biotechnology 4:520-525), but also yeast cells (Buckholz R.G., 1993, Yeast Systems for the Expression of Heterologous Gene Products. Curr. Op. Biotechnology 4:538-542), as well as animal cells, in particular mammalian cell cultures (Edwards C.P. and Aruffo A., 1993, Current applications of COS cell based transient expression systems. Curr. Op. Biotechnology 4, 558-563) but also insect cells in which methods may be used involving for example baculoviruses (Luckow V.A., 1993, Baculovirus systems for the expression of human gene products. Curr. Op. Biotechnology 4, 564-572).

The invention comprises, in addition, the use according to the invention, characterized in that said antigen or hapten is coupled or mixed with said membrane fraction,

in particular covalently coupled with at least one of the compounds contained in the membrane fraction.

In a preferred embodiment, the invention comprises the use according to the invention, characterized in that said antigen or hapten is covalently coupled with a supporting peptide to form a complex capable of binding specifically to mammalian serum albumin, preferably said supporting peptide is a peptide fragment derived from streptococcal G protein, in particular the C-terminal fragment called BB.

Of course, said complex may be prepared by genetic recombination.

The chimeric or hybrid complex may be produced by recombinant DNA techniques by insertion or addition of a sequence encoding said antigen or hapten of a protein nature to a DNA sequence encoding said peptide fragment of the streptococcal G protein.

The methods for the synthesis of hybrid molecules include the methods used in genetic engineering for constructing hybrid polynucleotides encoding the desired polypeptide sequences. Reference may be advantageously made, for example, to the technique for producing genes encoding fusion proteins which is described by D.V. Goeddel (Gene expression technology, Methods in Enzymology, Vol. 185, 3-187, 1990).

According to the present invention, the covalent coupling may be carried out by chemical synthesis. In a particular embodiment of the invention, it will be possible for one or more linking elements to be introduced into at least one of the compounds contained in the membrane fraction and/or in said antigen or hapten to facilitate the chemical coupling.

Preferably, said linking element introduced is an amino acid.

According to the invention, it is possible to introduce one or more linking elements, in particular amino acids, to facilitate the coupling reactions between a compound of the membrane fraction, and said antigen or hapten. The covalent coupling between said compound of the membrane fraction and said antigen or hapten according to the invention may be achieved at the N- or C-terminal end of said compound of the membrane fraction or of said antigen or hapten, if the latter are for example of a peptide nature. The bifunctional reagents allowing the coupling will be determined according to the end which is chosen for the coupling and the nature of said antigen or hapten to be coupled.

The invention also comprises the use according to the invention, characterized in that the coupling between said antigen, hapten or complex and at least one of the compounds contained in the membrane fraction is carried out by genetic recombination when said antigen, hapten or complex and said membrane compound are of a peptide nature.

The coupling between said antigen, hapten or complex, and at least one of the compounds contained in the membrane fraction may indeed be carried out by genetic recombination. It will be possible, for example, before extracting its membrane fraction, to transform said gram-negative bacterium beforehand with a vector containing a nucleic construct encoding an antigen of interest or said complex, such that the bacterium thus transformed expresses the antigen of interest or said complex attached to the membrane or anchored in the membrane of said bacterium. Such methods for expressing recombinant proteins attached to the membrane are well known and require, for example, the presence of a

specific regulatory sequence, such as a signal peptide-type sequence.

The subject of the invention is also the use according to the invention, characterized in that the pharmaceutical composition comprises, in addition, an agent which makes it possible to carry said membrane fraction associated with said antigen, hapten or complex in a form which makes it possible to enhance its stability and/or its immunogenicity, such as in the form of an oil-in-water or water-in-oil type emulsion, or in the form of a particle of the liposome, microsphere or nanosphere type or any type of structure allowing the encapsation and the presentation, in particulate form, of said membrane fraction associated with said antigen, hapten or complex.

The invention also relates to the use according to the invention, characterized in that said agent is chosen from aluminum salts, calcium salts, compounds of plant origin such as Quil A or saponin, or compounds of bacterial origin such as the derivatives of cholera, pertussis or tetanus toxoid or of the E. coli thermolabile toxin.

Also included in the present invention is the use according to the invention, characterized in that the pharmaceutical composition comprises, in addition, an agent which makes it possible to regulate the immune response induced by said membrane fraction combined with said antigen, hapten or complex.

Among said regulatory agents, cytokines, growth factors, hormones or cellular components such as nucleic acids, a protein of the family of heat shock proteins or ribosomes are in particular preferred.

The subject of the invention is also the use according to the invention, for the preparation of a

pharmaceutical composition intended for the prevention or treatment of infectious diseases of viral, bacterial, fungal or parasitic origin, or for the prevention or treatment of cancers, in particular cancers in which the tumors are associated with tumor antigens.

Among said infectious diseases of viral origin, the infectious diseases caused by paramyxoviruses, in particular by the parainfluenzae virus and more preferably by the respiratory syncytial virus (RSV) are particularly preferred.

In a particular embodiment, the use according to the invention is characterized in that said antigen associated with the membrane fraction comprises the peptide G2Na, a fragment of the G protein of the virus having an amino acid sequence SEQ ID No. 4, a peptide homologous to G2Na whose sequence exhibits at least 80%, preferably 90%, 95% and 99% identity, after alignment with the sequence SEQ ID No. 4, or the peptide G2Na or one of its homologs, covalently coupled with a C-terminal fragment (BB) of the streptococcal G protein to form a complex capable of binding to mammalian serum albumin, peptide BB as described in the documents Power et al., 1997 (Virology, 230, 155-166) and WO 96/14416.

The expression "percentage, degree or level of identity" between two nucleic acid or amino acid sequences for the purposes of the present invention is understood to mean a percentage of identical nucleotides or amino acid residues between the two sequences to be compared, obtained after the best alignment, this percentage being purely statistical and the differences between the two sequences being randomly distributed over their full length. The sequence comparisons between two nucleic acid or amino acid sequences are traditionally carried out by

comparing these sequences after having aligned them in an optimum manner, said comparison being carried out by segment or by "comparison window" to identify and compare the local regions of sequence similarity. The optimum alignment of the sequences for the comparison may be carried out either manually or by means of the local homology algorithm by Smith and Waterman (1981) [Ad. App. Math. 2:482], by means of the local homology algorithm by Neddleman and Wunsch (1970) [J. Mol. Biol. 48:443], by means of the method of search for similarity by Pearson and Lipman (1988) [Proc. Natl. Acad. Sci. USA 85:2444], by means of computer software using these algorithms (GAP, BESTFIT, FASTA and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI, or by the comparison software packages BLAST N or BLAST P).

The percentage identity between two nucleic acid or amino acid sequences is determined by counting these two sequences optimally aligned by the comparison window in which the region of the nucleic acid or amino acid sequence to be compared may comprise additions or deletions relative to the reference sequence for an optimum alignment between these two sequences. The percentage identity is calculated by determining the number of identical positions for which the nucleotide or the amino acid residue is identical between the two sequences, by dividing this number of identical positions by the total number of positions in the comparison window and by multiplying the result obtained by 100 in order to obtain the percentage identity between these two sequences.

For example, it will be possible to use the BLAST program, "BLAST 2 sequences", available on the site http://www.ncbi.nlm.nih.gov/gorf/bl2.html, the parameters used being those given by default (in particular for the parameters "open gap penaltie": 5, and "extension gap penaltie": 2; the template chosen

being for example the template "BLOSUM 62" proposed by the program), the percentage identity between the two sequences to be compared being calculated directly by the program.

In another aspect, the invention relates to a method for preparing a membrane fraction of gram-negative bacteria, in particular Klebsiella *pneumoniae*, characterized in that it comprises the following steps:

a) culture of said bacteria in a culture medium allowing their growth followed by centrifugation of said culture;

b) where appropriate, deactivation of the lytic enzymes of the bacterial pellet obtained in step a), followed by centrifugation of the suspension obtained;

c) extraction and removal of nonmembrane proteins and of nucleic acids from the pellet obtained in step a) or b) by at least one cycle of washing the pellet in an extraction solution;

d) digestion of the membrane pellet obtained in step c) in the presence of protease enzymes, followed by centrifugation;

e) at least one cycle of washing of the pellet obtained in step d) in physiological saline and/or in distilled water; and

f) ultrasonication of the pellet obtained in step e).

The invention also comprises the method for preparing a membrane fraction of gram-negative bacteria, in particular Klebsiella *pneumoniae*, characterized in that it comprises the following steps:

a) culture of said bacteria in a culture medium allowing their growth, followed, where appropriate, by centrifugation;

b) freezing of the culture medium or of the pellet obtained in step a) followed by thawing and drying of the cells;

c) removal, by means of a DNase, of the nucleic acids from the dry cells obtained in step b) which have been resuspended;

d) grinding of the cells obtained in step c) and clarification of the suspension obtained;

e) precipitation, in an acid medium, of the suspension obtained in step d) and removal of the pellet;

f) neutralization of the supernatant obtained in step e) containing the membrane suspension, followed by dialysis and concentration of the membrane suspension; and

g) sterilization of the concentrated membrane suspension obtained in step f).

The membrane fractions capable of being obtained by said methods indeed form part of the invention.

The content of proteoglycan of the membrane fractions capable of being obtained by said methods, an active ingredient of FMKp, represented by the sum of the contents of galactose and of protein, is preferably between:

- for galactose: between 1.2 g/l and 3.4 g/l;
- for the proteins: between 7.5 g/l and 14.9 g/l.

More preferably, this content will be:

- for galactose: between 1.6 g/l and 2.6 g/l;
- for the proteins: between 9.3 g/l and 11.7 g/l.

The invention relates, in addition, to the pharmaceutical compositions comprising a membrane fraction capable of being obtained by the methods according to the invention, preferably, said

pharmaceutical compositions comprise, in addition, an antigen, a hapten or a complex, as defined above, associated with said membrane fraction, such as in particular viral antigens or complexes specific to paramyxoviruses, or the antigens associated with tumor cells.

Of course, said pharmaceutical compositions according to the invention may comprise, in addition, the agents such as the vehicles and the regulatory agents defined above.

In a preferred embodiment, the pharmaceutical composition according to the invention is characterized in that said antigen associated with the membrane fraction comprises the peptide G2Na having the sequence SEQ ID No. 4 of the respiratory syncytial virus, one of its homologs as defined above, said peptide G2Na, or one of its homologs, covalently coupled with a C-terminal fragment (BB) of the streptococcal G protein to form a complex capable of binding to mammalian serum albumin.

The legend to the figures and examples which follow are intended to illustrate the invention without in any way limiting the scope thereof.

Legend to the figures:

Figure 1: BBG2Na adjuvanted with FMKp - dose-response study (serum anti-G2Na IgG titers).
* $p < 0.05$ (compared with the PBS group).

Figure 2: BBG2Na adjuvanted with FMKp - anti-G2Na IgG1 and IgG2a titers.

Figure 3: BBG2Na adjuvanted with FMKp - protection study.

Figure 4: Adjuvant effect of FMKp toward Immugrip (influenza vaccine).

* $p < 0.05$ compared with the nonadjuvanted group ("0") on the same day of sample collection.

Example 1: Production of the membrane fraction of *K. pneumoniae* (FMKp)

Method No. 1

The extraction of the K. pneumoniae I145 membranes from the centrifugation pellet of the step is preferably preceded by a step of destroying the lytic enzymes of the cellular components obtained in the pellet, for example by heating the latter at 100°C, optionally after redissolving in solution.

The actual extraction of the membranes from the centrifugation pellet is preferably carried out by treating the cellular components of the pellet, after optional destruction of the lytic enzymes, with a saline solution, for example 1 M sodium chloride, once or several times, followed by centrifugation, preferably at 20,000 g, of the suspension obtained, the supernatant from this centrifugation, which is eliminated, contains nonmembrane impurities such as proteins and nucleic acids, while the pellet contains the membranes.

After separation of the saline solution containing the impurities, the membranes are digested in the presence of proteolytic enzymes, preferably trypsin and chymotrypsin, in solution at pH 8 at 37°C for 4 hours.

After digestion, the solution is homogenized by ultrasonication. The product thus obtained constitutes the membrane fraction called FMKp.

The supernatant obtained is again centrifuged under the same conditions, preferably at 140,000 g.

Preparation of the membrane glycopeptides

This fraction is prepared from the pellet obtained by centrifugation at 40,000 g for 20 minutes. Said pellet is resuspended in physiological saline and then this suspension is heated for 10 minutes at 100°C on a boiling water bath to inactivate the lytic enzymes. After cooling, the medium is centrifuged for 30 min at 20,000 g. The pellet obtained is extracted twice with 1M NaCl in order to eliminate the proteins and the nucleic acids. The membranes are recovered by centrifugation for 30 minutes at 20,000 g.

They are then subjected to digestion with trypsin at pH 8 and at 37°C for 4 hours and then with chymotrypsin under the same conditions.

The membranes are then recovered by centrifugation at 2000 g for 30 minutes, washed with physiological saline and then with distilled water and are subjected to disintegration by ultrasound for 15 minutes.

Method No. 2

After thawing at +4°C for 48 h minimum, 1 kg of dry K. pneumoniae cells is resuspended in solution at 5% dry cells. The DNase is added at 5 mg/l. Grinding in a Manton Gaulin loop is then carried out for 30 min, followed by clarification on SHARPLES at 50 l/h, followed by precipitation with acetic acid at pH = 4.2 + 0.1 for 30 min. The pellet is removed (SHARPLES at 25 l/h) and the supernatant is neutralized, diluted to twice the initial volume with osmosed water. Constant-volume dialysis is then carried out on PUF 100 up to 800 Ωcm, followed by concentration of the membrane suspension (MS) thus obtained, to 11 l/kg of dry cells. The MS is then autoclaved at +121°C for 35 min and preserved at +4°C for 6 weeks.

Characteristics of the FMKp

By definition, the content of proteoglycan, an active ingredient of FMKp, is equal to the sum of the contents of galactose and of proteins.

- Galactose: on average 2.2 g/l
- Proteins: on average 10.5 g/l.

Example 2: Adjuvant effect of FMKp on a recombinant protein, BBG2Na

BBG2Na is a recombinant protein produced in E. *coli*. It consists of the peptide G2Na having the sequence SEQ ID No. 4, the fragment of the G protein of the respiratory syncytial virus (RSV) type A extending from residue 130 to residue 230, fused with BB, a fragment of the streptococcal G protein, having the capacity to bind to serum albumin. BBG2Na is an anti-RSV vaccine (Power U. Virology 1997, 230:155-166).

BALB/c mice receive 2 subcutaneous injections of 20 µg of BBG2Na and various quantities of FMKp. Blood samples are collected on D28 and the serum antibody titers are determined by ELISA with G2Na in solid phase. The results obtained are illustrated by figure 1. Surprisingly, they show that FMKp significantly increases the anti-G2Na IgG response; the anti-G2Na IgG titer reached is similar to those induced by alum or Freund's adjuvant. The effect is dose-dependant: it is observed from 5 µg of FMKp, is maximum from 50 µg of FMKp and remains stable with 100 µg of FMKp. FMKp is therefore a potential adjuvant for BBG2Na.

To know the effect of FMKp on the orientation of the immune response, in terms of Th1/Th2 response, the anti-G2Na IgG1 and IgG2a titers were determined on sera obtained as specified above. The results (figure 2) show that, surprisingly, FMKp is capable of modifying

the anti-G2Na IgG1/IgG2a ratio, in contrast to that which is observed with alum, for which the predominant isotype is IgG1. This profile is close to that induced by Freund's adjuvant. This indicates that FMKp may be used as immunity adjuvant to induce a mixed (Th1/Th2) type response.

The animals immunized as described above received a viral challenge by the nasal route with 10^5 $TCID_{50}$ of RSV-A. This was carried out 3 weeks after the last immunization. Five days after the viral challenge, the animals were sacrificed and the lungs removed in order to determine the RSV-A titers. The results (figure 3) show that the animals which received BBG2Na adjuvanted with FMKp are protected against an RSV-A challenge.

In conclusion, FMKp makes it possible to reorient the antibody response without affecting the capacity to protect mice against an RSV-A challenge.

Example 3: Adjuvant effect of FMKp on an inactivated virus (influenza vaccine)

BALB/c mice receive a single injection of 0.01 µg of Immugrip™ (influenza vaccine marketed by Laboratoires INAVA), and various quantities of FMKp. The products are coadministered. The injection is performed subcutaneously at D0. Blood samples are collected at D7, D14 and D21. The anti-Immugrip serum IgG antibody titer is determined by ELISA with Immugrip at 2 µg/ml in solid phase. The results presented (figure 4) show that FMKp significantly increases the anti-Immugrip antibody titer, this being from the lowest dose of FMKp, namely 0.1 µg. The adjuvant effect is dose-dependant. It is observed, interestingly, that the presence of FMKp induces the generation of an earlier antibody response, obtained from D7, compared with the nonadjuvanted Immugrip control. This effect is not

obtained with the reference adjuvant, complete Freund's adjuvant (CFA).